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High-level generation of polyclonal antibodies by genetic immunization

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Antibodies are important tools for investigating the proteome, but current methods for producing them have become a rate-limiting step[1]. A primary obstacle in most methods for generating antibodies or antibody-like molecules is the requirement for at least microgram quantities of purified protein. We have developed a technology for producing antibodies using genetic immunization[2]. Genetic immunization–based antibody production offers several advantages, including high throughput[3] and high specificity. Moreover, antibodies produced from genetically immunized animals are more likely to recognize the native protein[2]. Here we show that a genetic immunization–based system can be used to efficiently raise useful antibodies to a wide range of antigens. We accomplished this by linking the antigen gene to various elements that enhance antigenicity and by codelivering plasmids encoding genetic adjuvants. Our system, which was tested by immunizing mice with >130 antigens, has shown a final success rate of 84%.

Genetic immunization has received relatively little attention as a method for producing antibodies for proteomic applications. One reason has been the variable success of genetic immunization in producing antibodies[4]. To develop improved genetic immunization plasmids for more reliable generation of antibodies, we began with plasmid pBQAP10 (**Fig. 1**), which encodes a secretion leader sequence from the highly expressed human gene encoding α1-antitrypsin (AAT). Many studies have shown that adding a secretion leader sequence can markedly increase the antibody response[5,6]. After the leader sequence is a unique 20–amino acid antigenic tag that we included as an internal control. Secretion of the antigen may be blocked by 'quality control' if it is poorly folded or insoluble[7]. To improve protein solubility, we included a highly soluble and stably folded domain from the rat cartilage oligomerization matrix protein (COMP)[8]. The 46-residue COMP domain can also form pentamers and may enhance antigen uptake by antigen-presenting cells or allow T helper–independent B-cell activation[9,10].

We used the human gene encoding AAT as an antigen to test the efficacy of pBQAP10 in genetic immunization. Many different cytokines had earlier been tested as genetic adjuvants, with mixed results[11]. Plasmids expressing granulocyte/macrophage colony-stimulating factor (GM-CSF) have been widely used in genetic immunization studies and almost always result in an increase in antibody titer[11]. GM-CSF is a potent growth factor for dendritic cells, although its exact mechanism of action in genetic immunization is poorly understood. We immunized mice with the AAT-expressing plasmid using a gene gun, either with or without coadministration of plasmids encoding the cytokines GM-CSF and FMS-like tyrosine kinase 3 ligand (Flt3L)[12]. ELISA measurements of sera showed that the mice coimmunized with both the GM-CSF and Flt3L plasmids had approximately a ninefold higher antibody titer (3×10^4 titer, **Fig. 2a**). For comparison, a group of mice immunized conventionally using AAT protein with Freund's complete adjuvant produced antibody titers of 7×10^4. All genetically immunized mice responded with relatively little variation in titers (**Fig. 2b**). Isotyping of the AAT antibodies showed only the IgG_1 isotype (data not shown). The specificity of the sera was tested by probing a western blot containing AAT mixed with an *Escherichia coli* whole-cell extract. Pooled sera from five mice recognized a single band of the correct size for AAT (**Fig. 2c**).

To evaluate the general usefulness of this antibody production system, we tested it using a set of 100 antigen genes (see **Supplementary Table 1** online). Of the 100 genes tested, 36% encoded fragments of the mature form of the protein. The average identity of the human antigens to mouse proteins was 76%, and the average antigen size was 179 residues. Most of the genes were of human origin, and we explored three general sources of antigen genes: genomic DNA (20 genes), cDNA (52) and gene synthesis from oligonucleotides (28). In principle, amplifying genes from genomic DNA is the simplest approach because only a single template and two PCR primers are required per gene, or four primers for nested PCR. Genes fragmented into small exons may present a problem. For example, genes in the human genome are on average broken into 8.8 exons encoding an average length of 50 residues[13]. Using cDNA would bypass this problem but is more difficult logistically. Both genomic DNA and cDNA have the disadvantage that the genes may contain suboptimal codon usage. Codon optimization of genes has been shown to markedly increase translation and, as a consequence, antibody responses[14,15]. Gene synthesis allows codons to be optimized for expression and gives unrestricted access to any gene sequence. We recoded genes using a subset of codons allowing efficient expression in both mice and *E. coli* (see Methods).

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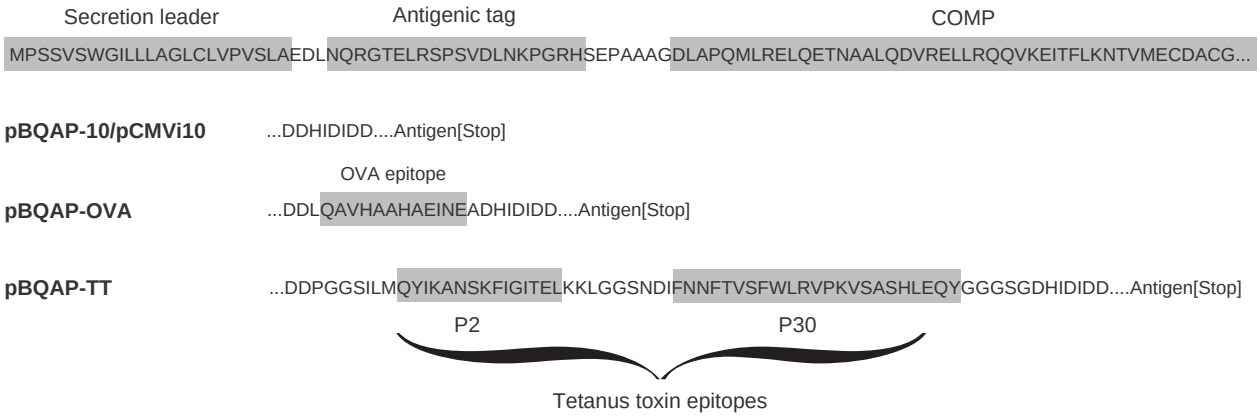

Figure 1 Design of genetic immunization vector. The plasmids pBQAP10, pBQAP-OVA and pBQAP-TT all contained the SP72 promoter and the rabbit β-globin terminator flanking the expression cassette shown above. The pCMVi10 plasmid is identical to pBQAP10 except that it contains the CMV promoter. The sequence HIDIDD is encoded by the 5′ flanks included in the PCR primers used to amplify the antigen gene.

PCR products of the 100 antigen genes were generated using primers with a flanking sequence containing deoxyuracil (dU) residues allowing rapid cloning[16]. The genes were cloned into either pBQAP10 (80 genes) or pCMVi10 (20) to allow genetic immunization of mice and into pGST-FRP for overexpression in *E. coli*. Overexpression was obtained successfully in *E. coli* with 88 of the 100 proteins. Groups of two CD1 mice were immunized and were boosted every 3 weeks until a total of four shots had been administered. Sera from mice were tested every 3 weeks by western blotting and were scored successful if 50 ng of the antigen was detected at sera dilutions of 1:5,000. Antibodies were detected against 62 of the 88 test antigens (70%) and were produced after an average of two immunizations (**Supplementary Table 1** and **Supplementary Fig. 1** online). The pBQAP10 and pCMVi10 vectors had similar efficacies.

Antigens that have high identity to sequences from the immunized host typically do not produce an antibody response as a result of tolerance mechanisms[17]. Analysis of the antigens tested in pBQAP10 and pCMVi10 indicated that this may indeed be a limitation, because antigens that failed to produce an antibody response had on average a higher identity to a mouse protein than successful antigens (69% versus 61%; **Supplementary Table 1**). Humoral tolerance can be overcome by adding exogenous T-cell epitopes fused to the antigen[18,19]. To evaluate this idea we created two new vectors, pBQAP-TT and pBQAP-OVA (**Fig. 1**), that contained either the P2 and P30 'universal' T-cell epitopes and flanking regions from tetanus toxin (50 residues) or the ovalbumin(325–336) T-cell epitope (12 residues).

A set of 38 gene fragments was cloned into either pBQAP-TT or pBQAP-OVA (see **Supplementary Table 2** online). Most of the genes encoded proteins that were expected to be poorly antigenic, because they were small (≤20 amino acids), were highly identical to mouse sequences (≤100%) or had earlier failed using protein-based immunizations. In addition, we included five genes that had earlier failed to yield antibodies in genetic immunizations when cloned in pBQAP10. The target region of each gene was selected on the basis of its antigenicity index score[20]. On average, the antigens contained 73 amino acids and had a 90% identity to a mouse protein.

Protein was successfully overproduced in *E. coli* for 97% of the genes. Antibodies were produced after an average of two immunizations. Antigens identical to mouse sequences were as successful as antigens with lower identity, and there was no substantial difference in success rate between the two T-cell epitope vectors. Others have reported producing antibodies against self-proteins by fusing T-cell epitopes[18,19], and we have shown that this approach seems to work with many self-proteins. Four of the five antigens that earlier failed to induce antibodies in pBQAP10 now produced antibodies. Furthermore, four antigens that earlier failed to produce antibodies when delivered as protein now produced antibodies (ApoAV, R26W, RYR2, Ub). Overall, 87% of large antigens (≥70 residues) and 79% of the small antigens (≤20 residues) produced antibodies, with an overall success rate of 84% (**Supplementary Table 2** and **Supplementary Fig. 1**).

There are few published studies with which the antibody production method developed in this study can be compared. The largest study to date is one that used protein immunizations with 570 antigens from *Neisseria meningitidis*[21]. Only 350 of the proteins could be overexpressed in *E. coli*, and of those, only 85 (24%) produced "strongly positive" antibodies. Another large study with a set of 40 synthetic peptides linked to keyhole limpet hemocyanin obtained a 63% success rate[22].

To investigate possible causes of failure in our system, we tested sera for antibodies against the antigenic tag. All of eight sera with antibodies against the test antigen also contained antibodies against the tag. Of ten sera that did not contain antibodies against the test antigen, eight did contain antibodies against the tag. Therefore, we can eliminate many nonimmunological causes of antibody response failure such as suboptimal bullet preparation, plasmid delivery, protein translation and protein secretion. Remaining possible causes of failure include post-translational modification of the antigen, structural features of the antigen and B-cell unresponsiveness. Sera were also tested for antibodies against other regions of the scaffold. We did not detect antibodies to the COMP domain or to the tetanus toxin epitopes, and only one of seven samples had antibodies against the ovalbumin epitope (data not shown).

To examine whether the antibodies we produced were useful for measuring the natural antigen, we used 12 of the antibodies to probe biological samples in which the antigen was known to be expressed. All 12 antibodies detected a protein of the correct size in the appropriate sample, but not in a control sample (**Fig. 3**). Sensitivity was tested with randomly selected antibodies by titrating the corresponding glutathione *S*-transferase (GST) fusion proteins on a western blot. Most of the antibodies, including those raised against self-proteins, could detect as little as a few nanograms of the GST protein (**Supplementary Fig. 2**).

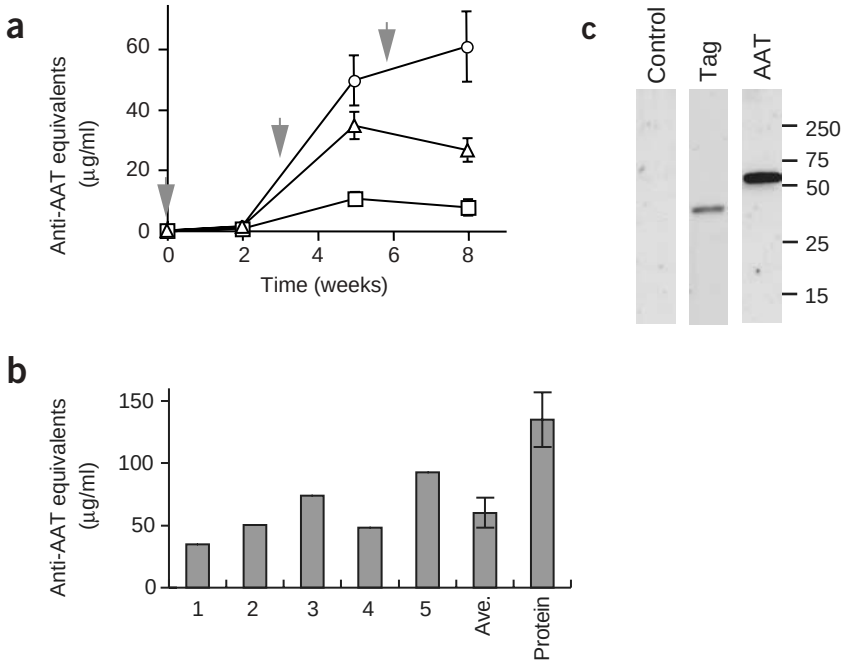

Figure 2 Antibody responses of mice immunized with pBQAP10-AAT. (**a**) Groups of five BALB/c mice were immunized with pBQAP10-AAT alone (□), with a GM-CSF plasmid (△) or with both GM-CSF and Flt3L plasmids (○). Antibodies to AAT were measured by ELISA and converted to monoclonal antibody equivalents using an anti-AAT monoclonal antibody of known concentration. The slopes of the curves for dilutions of the sera and the antibody were similar. Sera were diluted 1:250, 1:250, 1:1,000 and 1:6,000 for the 0, 2-, 5- and 8-week samples, respectively. Arrows indicate immunizations and bars standard errors. (**b**) Individual antibody concentrations measured by ELISA for five mice immunized three times with the plasmids containing genes encoding AAT, GM-CSF and Flt3L (AVE., average for these five mice) and a group of five mice immunized once with AAT protein. (**c**) Western blot analysis of sera pooled from five mice immunized as described in **a**. Control lane contains 10 μg of a whole-cell extract from *E. coli* with 50 ng of a GST fusion protein unrelated to AAT. The AAT and tag lanes are the same as the control lane, except for the addition to the samples before electrophoresis of 50 ng of pure AAT and 50 ng of GST tag, respectively. Sera were diluted 1:5,000.

Although antibodies were obtained against ≤84% of the gene products that could be expressed in *E. coli*, a number of caveats should be mentioned. First, protein synthesis in at least one system is required to test these antibodies. Although the proteins do not have to be purified, a great advantage over alternative methods, they do have to be made, because specificity cannot be confirmed without a protein source. If this is considered, the success rate is somewhat reduced to 82% for the small, difficult antigens expressed with T-cell epitopes, and 62% for the antigens expressed without the T-cell epitope. Overall, 90% of the 133 different antigens were successfully overexpressed in *E. coli*. This is a higher success rate than reported by other large-scale expression studies[21,23]. This higher success rate may largely be attributed to selecting small soluble fragments of proteins as well as avoiding membrane proteins or at least the membrane-associating region. Membrane proteins are typically the most difficult to overexpress, and it should be noted that half of the proteins that we failed to express in *E. coli* were membrane proteins. Moreover, 21% of the sera (**Supplementary Fig. 1**) showed some cross-reactivity with unexpected proteins in *E. coli* extracts supplemented with an irrelevant GST fusion protein. There is no indication that these sera will react with antigens from the same organism as the one used for genetic immunization; however, this finding shows a relatively high rate of spurious cross-reaction, which should always be borne in mind when testing these, or indeed any polyclonal, sera.

High-throughput genomic technologies currently produce complete genome sequences and allow the measurement of entire mRNA populations. Although these innovations have revolutionized biology, their impact will be limited unless the information generated can be translated to the protein level in a correspondingly high-throughput manner. We have developed a high-throughput system for generating antibodies that can help close the gap. Application of this system could range from small-scale analysis of interesting gene sets discovered by microarray analysis, to systematic generation of antibodies against all putative proteins discovered in genome sequencing projects. Each CD1 mouse yields ≤2 ml of serum, sufficient for hundreds of immunoassays. Spleens from the mice can be saved, allowing larger amounts of highly valuable antibodies to be generated later as monoclonal or single-chain antibodies[24,25].

METHODS

Construction of plasmids. The genetic immunization plasmids were derived from pCAGGS[26]. We replaced the human cytomegalovirus (CMV) promoter with a synthetic promoter, SP72. The SP72 element was designed *de novo* from consensus binding sites for transcription factors and rivals CMV in terms of producing antibody responses (B. Qu, University of Texas-Southwestern Medical Center, Dallas, Texas, USA, personal communication). A 618-bp fragment containing the SP72 promoter was subcloned at the *Sal*I and *Eco*RI sites, replacing the CMV promoter and intron, to create pSP72. Gene synthesis was used to construct a 346-bp DNA fragment containing, in the following order, an *Eco*RI site, a consensus translation initiation site, the leader sequence from *AAT*, the antigenic tag, COMP and restriction sites for *Bcl*I, *Xma*I and *Xba*I. The fragment was digested with *Eco*RI and *Xba*I and subcloned into the same sites in pSP72 to create pBQAP10. The plasmid pCMVi10 was identical except that it retained the original CMV promoter and intron. The plasmids pBQAP-OVA and pBQAP-TT were based on pBQAP10 and were created by subcloning a *Bgl*II- and *Xma*I-digested DNA fragment encoding the T-cell epitopes, and created by gene synthesis, into the *Bcl*I and *Xma*I sites. A new *Bcl*I site was designed after the T-cell epitope coding regions. The plasmid pGST-FRP was derived from pGST-CS[27] by subcloning a pair of annealed oligonucleotides at the *Nco*I and *Eco*RI sites. This replaced the existing multiple cloning sites for *Bgl*II, *Bam*HI and *Xma*I. The expression plasmids encoding GM-CSF and Flt3L were constructed by subcloning mouse cDNAs into pCMVi-SS[28] at the *Bgl*II and *Kpn*I sites.

Gene synthesis. Genes were designed with a set of codons selected for efficient expression in both mice and *E. coli*, and for design flexibility to avoid hairpins and other inappropriate matches among the sequence that can hinder gene synthesis. The codons used were as follow: Ala, GCA (33%), GCT (33%), GCC (34%); Cys, TGT (50%), TGC (50%); Asp, GAT (50%), GAC (50%); Glu, GAG (50%), GAA (50%); Phe, TTT (25%), TTC (75%); Gly, GGT (50%), GGC (50%); His, CAT (25%), CAC (75%); Ile, ATT (25%), ATC (75%); Lys, AAG (50%), AAA (50%); Leu, CTG (100%); Met, ATG (100%); Asn, AAC (100%); Pro, CCG (50%), CCA (50%); Gln, CAG (75%), CAA (25%); Arg, CGT (25%), CGC (75%); Ser, TCT (50%), AGC (50%); Thr, ACT (50%), ACC (50%); Val, GTG (75%), GTT (25%); Trp, TGG (100%); Tyr, TAT (50%), TAC (50%). A set

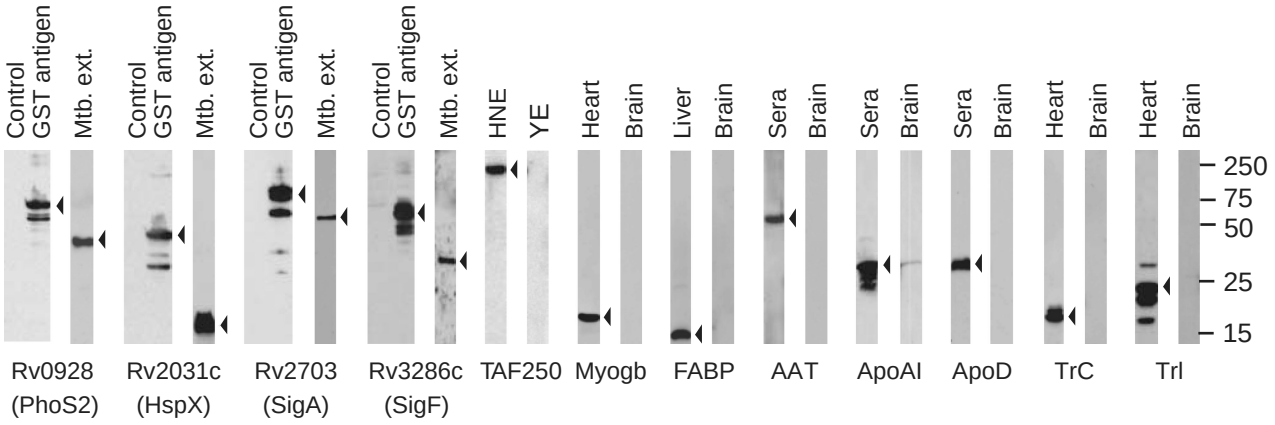

Figure 3 Western blot analysis of natural extracts. All antibodies were diluted 1:1,000. The antibodies raised against the Mtb proteins were used to probe western blots containing 3.25 μg of a *Mycobacterium tuberculosis* whole-cell extract (Mtb. ext.). As a control, the antibodies were used to probe a western blot containing 10 μg of an *E. coli* whole-cell extract with either 50 ng of an unrelated GST fusion protein (control) or the relevant GST antigen. The TAF250 antibody was probed against 4.5 μg of a HeLa cell nuclear extract (HNE) or 6 μg of a yeast extract (YE). The AAT, ApoAI and ApoD antibodies were probed against 7 μg of human sera or, as a control, 25 μg of a human brain extract. The myoglobin, FABP, TrC and Trl antibodies were probed against 25 μg of human brain, liver or heart extract. Arrows indicate the known sizes of the mature proteins.

of overlapping oligonucleotides was designed using the custom software DNABuilder (http://cbi.swmed.edu/computation/cbu). The oligonucleotides were assembled into a DNA fragment using PCR[29]. Genes were subcloned into the appropriate plasmids and sequenced to identify a correct clone. Mutations occurred at a frequency of 0.3%.

UDG cloning. PCR products were generated using primers containing 5′ flanks as described[16]. The forward primers contained the flanking sequence 5′-ATAUCGAUAUCGAUGAU-3′, and the reverse primers contained the flanking sequence 5′-AGUGAUCGAUGCATUACU-3′. Vector preparations were created by digesting the plasmids with *Bcl*I and *Xma*I (pBQAP10, pBQAP-OVA, pBQAP-TT) or *Bgl*II and *Xma*I (pGST-FRP), and ligating the following oligonucleotides to the 4-bp overhangs: 5′-GATCATATCGATATCGATGAT-3′ and 5′-CCGGAGTGATCGATGCATTACT-3′. PCR products were subcloned by mixing 50 ng of the vector preparation with 10 ng of the PCR product in the presence of 0.5 units of uracil DNA glycosylase (New England Biolabs), 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM dithiothreitol in a final volume of 10 μl. Reactions were incubated at 37 °C for 30 min, and 1 μl was used to transform *E. coli* DH10B.

Genetic immunization and analyses. All procedures for handling mice were approved by the University of Texas Southwestern Medical Center IACRAC. Plasmids were delivered using the Helios gene gun (Bio-Rad). Bullets were prepared according to the manufacturer's instructions with a mixture of plasmids encoding the antigen and plasmids encoding mouse GM-CSF and mouse Flt3L (2:1:1 ratio). Each bullet contained ~1 μg of DNA. Mice were anesthetized with avertin (0.4 ml/20 g mouse) and shot in each ear using 400 p.s.i. to fire the gene gun. Blood was collected by tail bleeds and allowed to stand for 2 h at 24 °C, after which the sera were collected by centrifugation. Western blots and ELISAs were done as described[3]. Each ELISA was done using an AAT monoclonal antibody as a standard (Calbiochem) to calculate antibody equivalents in micrograms per milliliter. Titers were defined as the reciprocal of the sera dilution that produced a signal twofold above background (age-matched sera). GST fusion proteins were generated in *E. coli* strain DH10B by inducing 2-ml log-phase cultures with isopropyl-β-D-thiogalactoside. Whole-cell extracts were prepared from bacteria 2 h after induction. Cells were pelleted, resuspended in 200 μl of PBS, mixed with 200 μl of SDS lysis buffer and heated for 5 min at 95 °C.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests

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